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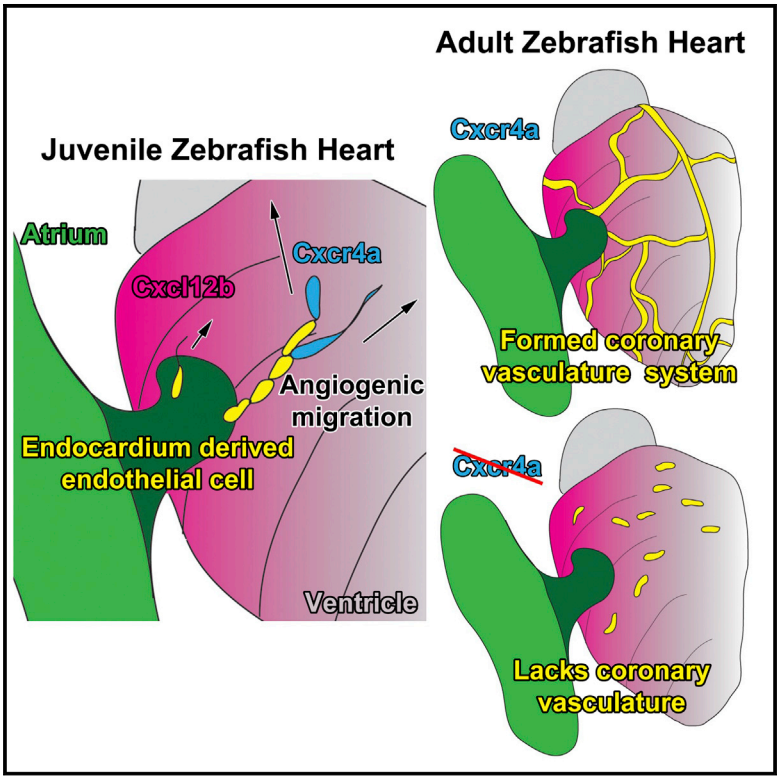
Developmental Cell

Chemokine-Guided Angiogenesis Directs Coronary Vasculature Formation in Zebrafish

Graphical Abstract



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In Brief

Harrison et al. describe coronary vessel formation in juvenile zebrafish by endocardial cells that migrate onto the ventricle. These endothelial cells sprout to create the vascular network under the guidance of chemokine signaling. Without these signals, the coronary vessels fail to form, resulting in reduced regenerative potential in adult zebrafish.

Highlights

- Zebrafish develop coronary vessels during late juvenile development
- Coronary vessel endothelial cells are derived from the endocardium
- Endothelial cells form coronary vessels under the guidance of CXC signaling
- *cxcr4a* mutant zebrafish hearts lack coronary vessels and are unable to regenerate

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Chemokine-Guided Angiogenesis Directs Coronary Vasculature Formation in Zebrafish

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SUMMARY

Interruption of the coronary blood supply severely impairs heart function with often fatal consequences for patients. However, the formation and maturation of these coronary vessels is not fully understood. Here we provide a detailed analysis of coronary vessel development in zebrafish. We observe that coronary vessels form in zebrafish by angiogenic sprouting of arterial cells derived from the endocardium at the atrioventricular canal. Endothelial cells express the CXC-motif chemokine receptor Cxcr4a and migrate to vascularize the ventricle under the guidance of the myocardium-expressed ligand Cxcl12b. *cxcr4a* mutant zebrafish fail to form a vascular network, whereas ectopic expression of Cxcl12b ligand induces coronary vessel formation. Importantly, *cxcr4a* mutant zebrafish fail to undergo heart regeneration following injury. Our results suggest that chemokine signaling has an essential role in coronary vessel formation by directing migration of endocardium-derived endothelial cells. Poorly developed vasculature in *cxcr4a* mutants likely underlies decreased regenerative potential in adults.

INTRODUCTION

The maintenance of systemic blood flow around the body places incredible demands on the heart so that it requires a continuous supply of oxygen and nutrients by an intricate network of coronary vasculature. Coronary disease is the leading cause of death worldwide (Alwan, 2011), and malformation of coronary vasculature can often cause sudden death in young adults (Taylor et al., 1992). Despite this critical importance, the processes and factors required for coronary vessel development remain to be elucidated.

The developmental origin of coronary vascular cells in birds and mammals has been an area of intense research and debate. Based on anatomical observations, coronary arteries were once thought to bud from the aorta in mammals (Hutchins et al., 1988). This was first questioned with experiments using chick-quail chimeras, which suggested that proepicardial cells spread over the heart and undergo EMT to differentiate and assemble into endothelial tubes (Pérez-Pomares et al., 2002). More recent work using mouse histological and clonal analyses suggest that proepicardial cells only make a small contribution to the coronary vasculature (Katz et al., 2012). Instead, vessels are thought to derive from existing endothelial cells that either sprout directly from the sinus venosus (SV) to cover and vascularize the heart (Chen et al., 2014; Red-Horse et al., 2010), form an intermediate subepicardial endothelial cell population that arises from the endocardium of SV and atrium (Tian et al., 2013) or derive from budding ventricular endocardial cells (Chen et al., 2014; Wu et al., 2012). The relative contribution of different sources appears to vary between different regions and vessels of the heart (Chen et al., 2014) and is complicated further by partial contributions of both endocardial and proepicardial cells to the SV prior to coronary vessel development (Katz et al., 2012; Wu et al., 2012).

Zebrafish have become a major model for the study of heart development and disease. Development of the zebrafish heart begins during gastrulation with the specification of endocardial and myocardial progenitor cells (Stainier et al., 1993). These progenitor cells undergo cardiogenic differentiation and heart morphogenesis after which a third cell layer, the epicardium, covers the outer surface (Figures S1B and S1F) (Serluca, 2008). The result is a fully functioning two contractile-chambered heart at hatching (at 5 days post-fertilization [dpf]) that supplies blood to the body via a single circulatory loop (Hu et al., 2000). Here, we report that coronary endothelial cells in zebrafish form by angiogenic sprouting from endocardial-derived cells during post-embryonic development and identify Cxcr4a-Cxcl12b signaling as a key regulator of this process.

CXC chemokines have a well-established role in regulating cell adhesion and migration during zebrafish development (Raz and

Mahabaleshwar, 2009). Zebrafish have two CXCL12 (also known as SDF1) encoding genes (*cxcl12a* and *cxcl12b*) and two receptors (Cxcr4a and Cxcr4b), which act as discrete pairs in the embryo to simultaneously direct cell migration during development (Boldajipour et al., 2011). Cxcl12a-Cxcr4b signaling is required for directed migration of individual germ cells or the collective migration of the lateral line primordium. In both cases, Cxcr4b-positive cells migrate along a Cxcl12a gradient (David et al., 2002; Doitsidou et al., 2002). Both axes of the signaling pathway are utilized in the step wise formation of the truck lymphatic network, where Cxcl12a first guides the dorsal migration of lymphatic progenitors then Cxcl12b guides the growth of the lymphatic sprouts along the intersegmental vessels (Cha et al., 2012). Independent Cxcl12b-Cxcr4a signaling has been implicated in the tethering of Cxcr4a-positive endodermal to the underlying Cxcl12b-experssing mesoderm during zebrafish gastrulation (Nair and Schilling, 2008) and later in the formation of the lateral dorsal aorta (Siekmann et al., 2009) and the capillary network of the zebrafish brain (Bussmann et al., 2011). Similarly, mouse knockouts of CXCR4 and CXCL12 show defects during embryonic vessel development, particularly in the gastrointestinal tract (Ara et al., 2005; Tachibana et al., 1998) and kidney (Takabatake et al., 2009). We have found that Cxcl12/Cxcr4 chemokine signaling is necessary for coronary vessel formation in zebrafish and is sufficient to provide the positional cues for this process. Furthermore, *cxcr4a* mutant hearts lose the potential to regenerate in adulthood, suggesting poorly developed vasculature in *cxcr4a* mutants likely underlies decreased regenerative potential in adult zebrafish.

RESULTS

In the 2 months following hatching, the zebrafish juvenile ventricular myocardium undergoes significant expansion (Figures 1A–1C; Figures S1A–S1L) (Gupta and Poss, 2012; Hu et al., 2000). Using a transgenic line that specifically labels endothelial cells (*fli1a:EGFP*), we observed that zebrafish did not develop coronary vasculature until after this expansion had started; they hatched without endothelial cells or vessels on the surface of the heart (Figures 1A–1C; Figures S1A–S1L). This is in striking contrast to sharks, birds, and mammals for which coronary vessel development occurs during embryogenesis (De Andrés et al., 1993; Hutchins et al., 1988; Pérez-Pomares et al., 2002; Red-Horse et al., 2010; Wu et al., 2012). The formation of coronary vasculature began 1-2 months post hatching (~14 mm in body length) with the emergence of endothelial cells between the atrium and ventricle (Figure 1C; Figures S1M–S1O). The newly emerged endothelial cells then migrated rostrally on to the bulbous arteriosus (BA) to form a vascular connection to the gills (Figure 1D; Figures S1P and S1R). This provides a supply of freshly oxygenated blood to the expanded myocardial layer via the main coronary artery over the BA which strongly expresses *kdrl:mCherry* (Figures 1D–1G), *dll4:EGFP* (Figures 1L and S1II; asterisk denotes artery) and *flt1:tdTomato* (Figures 1M and S1GG; asterisk denotes artery) indicative of its arterial fate (Bussmann et al., 2010; Torres-Vázquez et al., 2003; Wythe et al., 2013). As the heart continued to grow, angiogenic sprouts appeared to progressively spread over the ventricle in later juvenile stages (Figures 1E and 1F; Figures S1S–S1Z). The result was the formation of a dense network of vessels that covers the ventricle in adult zebrafish (Figure 1G; Figures S1AA–S1FF). The larval endocardium expresses arterial marker *dll4:EGFP* prior to the emergence to vessel sprouts (Figure 1I). These emerging sprouts also appear to be comprised of arterial endothelial cells (Figure 1J). As the coronary vasculature network forms, some of these endocardial-derived cells appear to progressively downregulate *dll4:EGFP* while others maintain a high level of expression. The result is the formation of a network of arteries (arrowheads) and non-arterial vessels (arrows) (Figures 1K–1M, S1GG, and S1II). Unlike other vasculature systems, such as in the fin, these non-arterial vessels do not appear to upregulate venous markers (Figures 1N and S1GG–S1LL), nor do they express lymphatic marker *prox1a:RFP* by this stage (113–127 dpf) (Figure S1KK).

The time of vessel emergence and rate of formation varied, but correlated strongly with age, fish length, and ventricle size when zebrafish were maintained under controlled conditions (Figure 1H; Figures S2G–S2I). Consistent with the stochastic nature of vessel emergence and the correlation between heart size and vessel area during development, the adult zebrafish coronary vasculature appears to respond to increasing heart size. Adult zebrafish that continued to grow had larger hearts and increased coronary vessel area and density (Figures S2A–S2F). Zebrafish raised at high density (20 fish per 3 L) until 8 months post-fertilization (mpf) and then switched to a lower density (four fish per 3 L) for 4 months became significantly larger and had a corresponding increase in heart size (Figures S2J and S2K). Coronary vessels of these fish appeared to adapt to these changes by increasing vessel area and vessel density despite their relatively old age (8–12 mpf, Figures S2L–S2O), demonstrating the dynamic nature of vessel development and its capability to adapt to environmental changes.

Endocardial Cells Migrate onto the Surface of the Ventricle

The progressive nature of vessel formation was indicative of an angiogenic process in which interlinked endothelial cells migrate over the heart to form interconnecting vessels. Time-lapse imaging of live juvenile hearts confirmed that endothelial sprouts actively migrated over the ventricle and made connections between endothelial cells (Figure 2A, arrows; Movie S1). To investigate the source of these angiogenic sprouts, we analyzed hearts at the earliest stage of vessel development at around 5 weeks post-fertilization (Figures 2B–2D). We consistently observed one or two *fli1a:EGFP* positive endothelial cells on the ventricle proximal to the atrioventricular (AV) canal at the groove between the two (Figures 2B and 2C). Cells in this position had direct connections to the endocardial layer, suggesting that they emerged from this point (Figure 2C′). In some cases, single cells spanned the thin myocardium while projecting processes across the surface of the heart (Figures 2D and 2D′). To test whether the endocardial endothelium was the source of vessel endothelial cells, embryonic endocardial cells were genetically labeled from 0 to 5 dpf. We generated clonal patches of mCherry-positive endocardial cells in the juvenile heart (prior to the emergence of coronary vessels) by

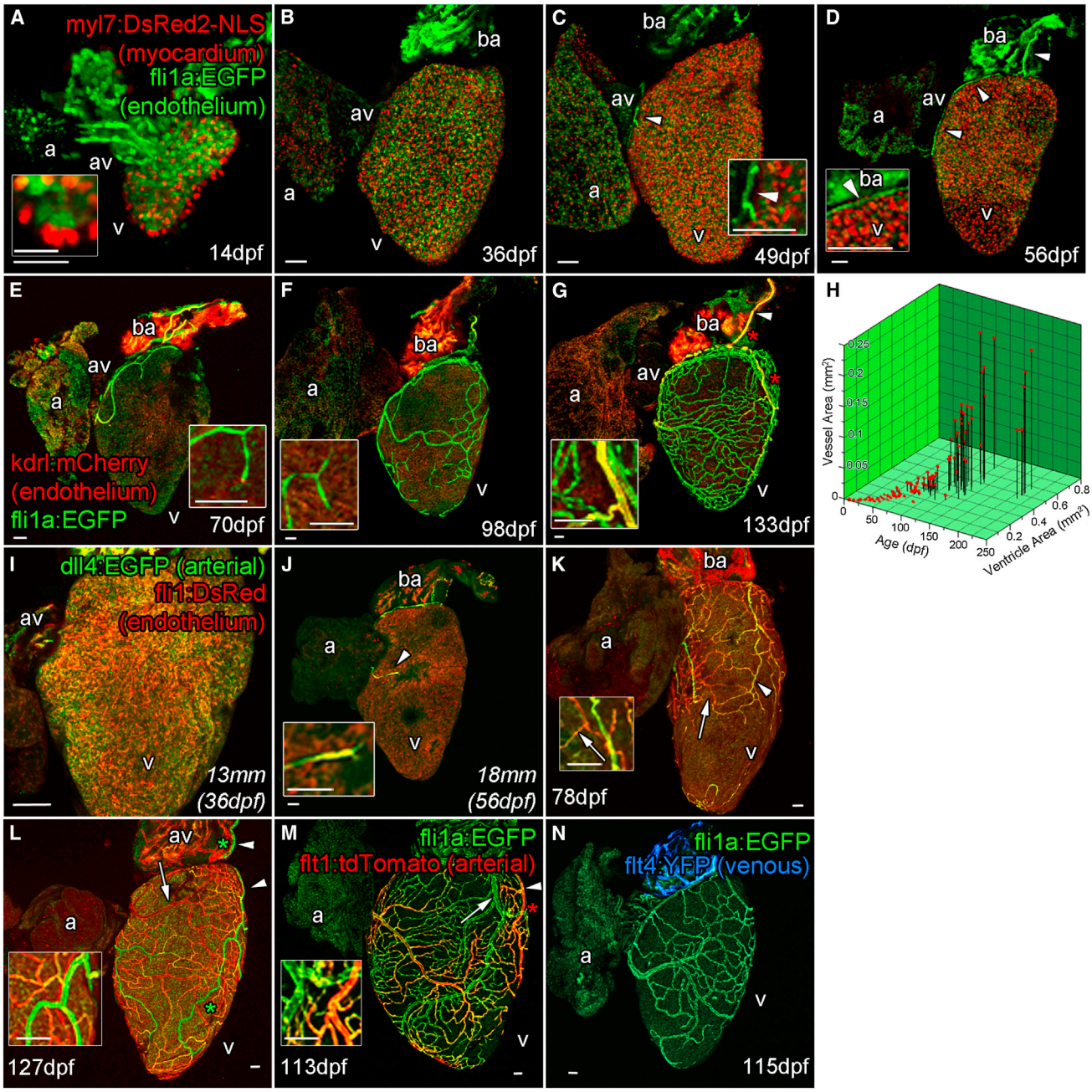

Figure 1. Zebrafish Coronary Vessels Develop during Juvenile Stages and Respond to Increased Heart Size

(A–G) Confocal images of whole larval, juvenile and adult double transgenic hearts, *Tg(fli1a:EGFP; myl7:DsRed2-NLS* (A–D) and *Tg(fli1a:EGFP; kdrl:mCherry)* (E–G). *flila:EGFP*-positive endothelial (endocardial) cells are visible only on the inside of the myocardium (labeled with *myl7:DsRed2-NLS*) and bulbous arteriosus after hatching (A and inset 14 dpf; and B, 36 dpf). Five to seven weeks later, endothelial cells are found on the surface of the heart (C, 49 dpf, arrowhead and inset), forming a vessel connection between the gills and AV-boundary after 1 month (D, 56 dpf, arrowheads and inset). The major vessel over the bulbous arteriosus expresses high levels of *kdrl:mCherry* (marked by arrowhead in G). Branches from these early vessels appear to progressively cover and interconnect over the juvenile heart into adulthood; however, the majority express low *kdrl:mCherry* levels (E, 70 dpf to G, 133 dpf, insets).

(H and I) The emergence and coverage of these vessels is stochastic, but correlates strongly with age and the area of the ventricle (H). Endocardial cells prior to the emergence of vessels express arterial marker *dll4:EGFP* (I).

(J and K) Emerged endothelial cells on the ventricle surface also have an arterial identity (J, arrowhead and inset), which is selectively downregulated in some vessels as they form (K, arrow).

(L and M) A subset of vessels maintain *dll4:EGFP* (L, arrowhead) and express *flt1:tdTomato* confirming they are arteries (M, arrowhead).

(N) Vessels that downregulate arterial makers appear to not progress to full venous identity as *flt4:YFP* expression is not observed at significant levels. a, atrium; av, AV canal; v, ventricle. Zebrafish age listed as dpf or italicized total body lengths with equivalent age in brackets when zebrafish were raised under non-standard conditions. *artery. Scale bars represent 50 μm except for that in (A, inset), which is 10 μm.

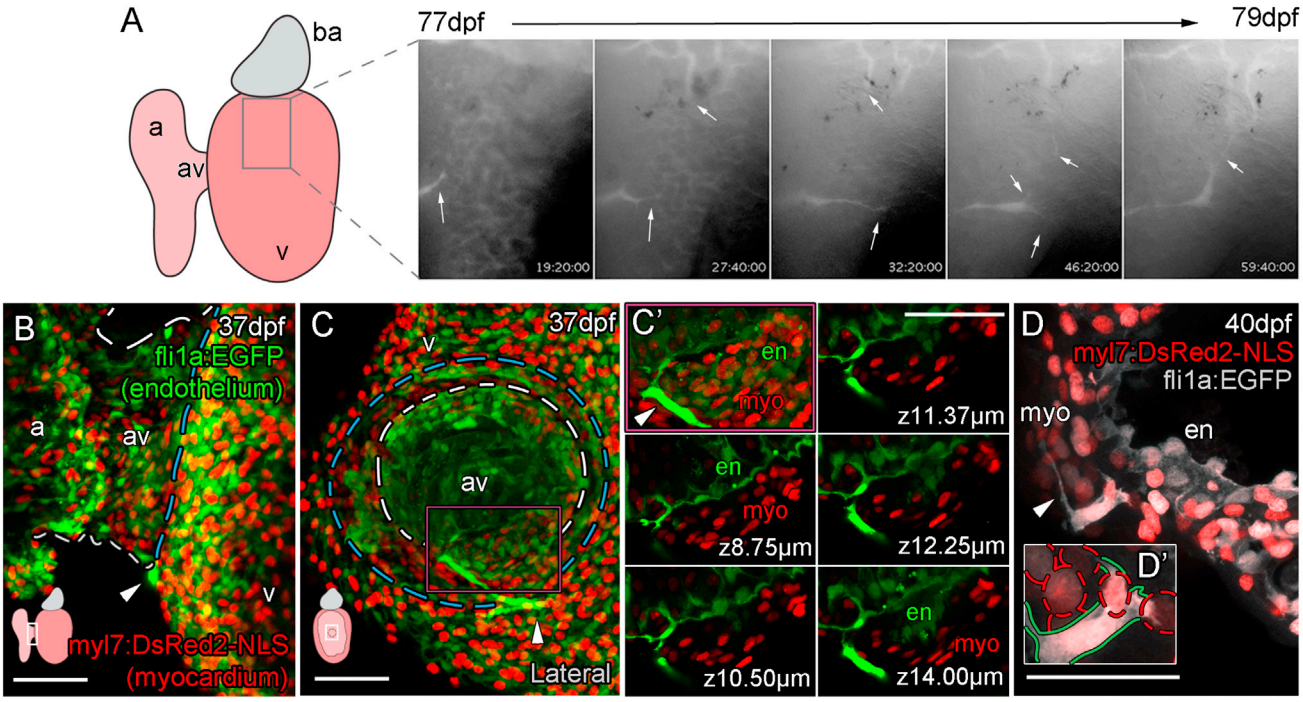

Figure 2. Vessels Are Formed from Angiogenic Sprouts of Cells Emerging from the AV Canal

(A–D′) Live imaging stills show the angiogenic migration of the endothelial cells that migrate over the ventricle surface and form interconnections between forming vessels (A; angiogenic sprouts marked by arrows). The first emerging endothelial cells are visible on the surface of the ventricle at the juncture of the atrium and ventricle (arrowheads, B, ventral view; C, lateral view of the same heart, diagram denotes heart orientation). Cells at this point have a direct connection to the endocardium (C, dashed white line demarks AV canal, blue line marks invagination of the ventricular myocardium; C′, higher magnification of region demarcated by purple box with select individual planes in z, labeled with distance from the first plane of projection). Individual endothelial cells span the myocardium (D; D′, higher magnification with red dashed lines demarcating myocardial nuclei, green line demarcates endothelial cell) and then project filopodia over the ventricle surface (D, arrowhead). Scale bars represent 50 μm.

inducing recombination of a transgenic reporter [*Tg*(*ubb:Lox-PEGFPLoxPmCherry*)] with a *fli1a* endothelial cell specific enhancer driven ERt2CreERt2 (Das and Crump, 2012) that was temporarily activated by 4-OHT in the embryo (Figures 3A and 3B; Figures S3A–S3I). Endothelial cells emerging from the AV canal in juvenile hearts were also mCherry-positive (39% of hearts with endocardium labeling, n = 23; Figures 3B, 3C, and S3G–S3I), suggesting that these cells were derived from embryonic endothelial cells, most likely from the labeled endocardium adjoining the AV canal (Figures 3C, S3G, and S3H). Live and fixed imaging suggested that vessels form through an angiogenic process, where endothelial cells divide and migrate over the heart (Risau, 1997) (Figures 1 and 2A; Figure S1; Movie S1). Consistent with these observations, the majority of developed vessels (*fli1a:EGFP* positive) on the adult heart were also continuously mCherry-positive along labeled vessels, indicating that they were derived from the early angiogenic sprouts observed in juvenile fish (Figure 3D, arrow; Figure 3E, no 4OHT control). Some unlabeled vessels were observed (Figure 3F, arrow) suggesting either that multiple endocardial cells transmigrate onto the surface of the ventricle, or that a secondary minor source of cells contributes to formation of these vessels. We quantified the contribution of the clonal patches to coronary endothelial cells and found that the majority of coronary endothelial cells were labeled in hearts with only partial endocardial labeling (9/23 labeled hearts, Figure S3I). We also found some hearts that had a large portion of the endocardium labeled, but lack vascular labeling (Figures S3E, S3F, and S3I). These data suggest that coronary endothelial cells were likely derived from a restricted population rather than many different regions of the endocardium. In addition, no vessel labeling was observed in hearts that, although treated with 4OHT, lack endocardial labeling due to the stochastic nature of recombination (n = 6, not included in quantification).

To further characterize the origin of coronary endothelial cells in zebrafish, we used a clonal analysis using the multispectral *ubb:zebrabow* line (*ubb:Lox2272-LoxP-RFP-Lox2272-CFP-LoxP-YFP*) and *kdrl:CreERt2* to label and then follow specific endocardial clones (Figures 3G–3L; Figures S3J–S3O; Movie S2) (Pan et al., 2013; Zhao et al., 2014). Embryonic induction (32–56 hours post-fertilization) of CreERt2 resulted in spectrally distinct patches of labeled endocardium in juvenile fish (Figures 3G–3L, arrowheads; Figures S3J–S3O). Endothelial sprouts observed on the surface of the heart often displayed the same spectral combination as endocardial clones within the AV canal (Figures 3G–3J, arrows; Figures S3M–S3O). Such endocardial spectral labeling often extends into the atrium, ventricle or both, but those restricted to the atrium or ventricle endocardium did not appear to give rise to emerging endothelial cells on the surface of the heart (Figures 3K and 3L; Figures S3M–S3O).

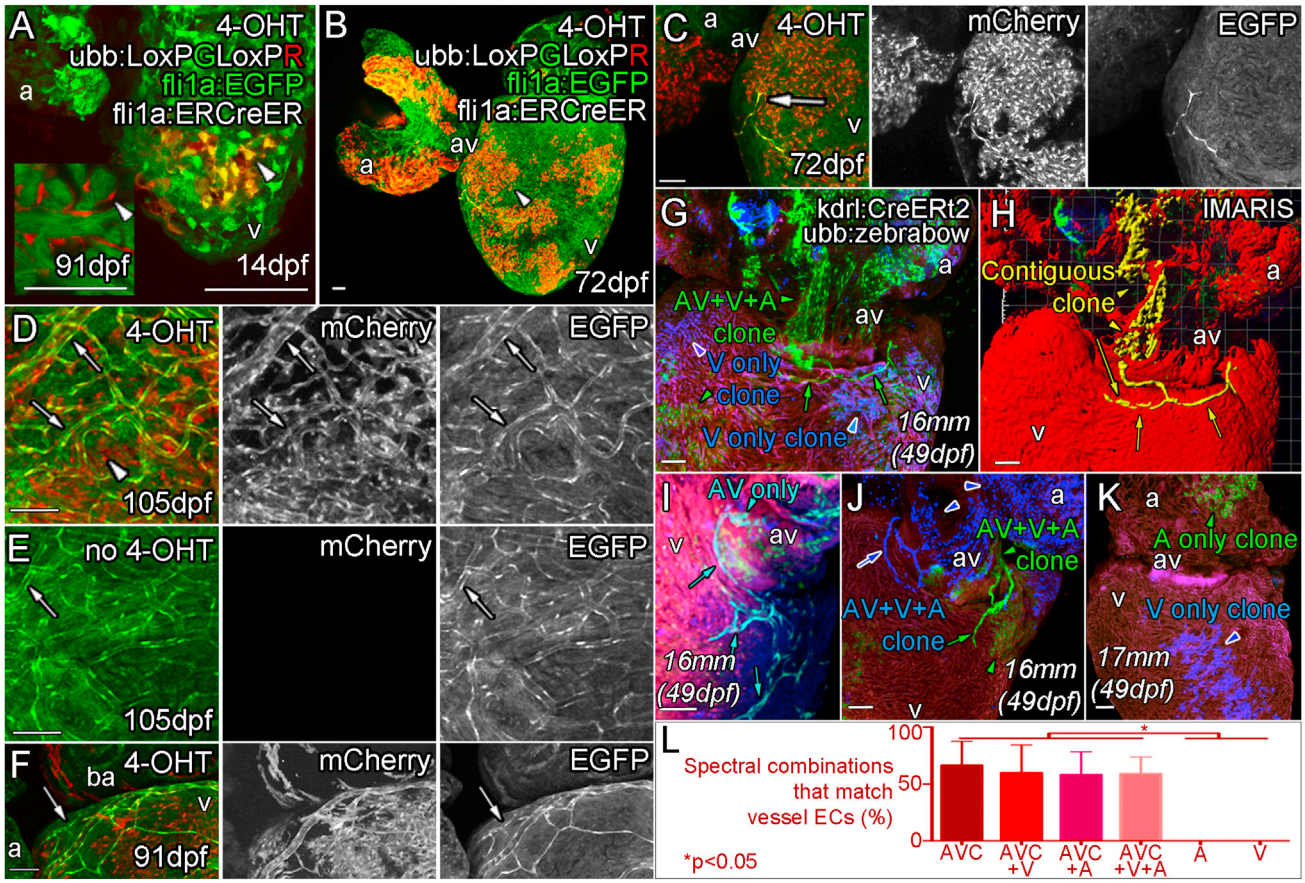

Figure 3. Angiogenic Coronary Endothelial Cells Are Derived from a Subpopulation of the Endocardium

Triple transgenic fish, *Tg (ubb:LoxPEGFPLoxPmCherry (ubb:LoxPGLoxPR); fli1aep:ERt2CreERt2 (fli1a:ERCreER); fli1a:EGFP)* were treated with 4-OHT during embryogenesis (0–5 dpf) to label the endocardium as mCherry positive clonal patches (arrowheads) and imaged at 14 dpf (A), 72 dpf (B and C), 91 dpf (F) and 105 dpf (D). (E) No 4-OHT control. (A, inset) 91 dpf section with no *fli1a:EGFP* showing mCherry expression in endocardial cells lining the trabecular myocardium. Emerging endothelial cells (*fli1a:EGFP*-positive, bright green) are also *mCherry*-positive (C and D, arrow, labeled endothelial cells; arrowhead, labeled endocardial cells). The majority of subsequently formed vessels are found to be similarly labeled in adult zebrafish treated with 4-OHT as embryos (D, arrows), but not in those that were not exposed to 4-OHT during embryogenesis (E, arrow). Unlabeled vessels (arrowhead) have been observed on hearts with labeled vasculature consistent with the hypothesis that multiple cells give rise to vessels by an angiogenic process (F, arrow). Clonal analysis with multispetral fluorescent proteins (*ubb:Zebrabow; ubb:Lox2272-LoxP-RFP-Lox2272-CFP-LoxP-YFP*) is used to follow the fate of restrictedly labeled endocardial cells (G–L). Image of the ventral side of the AV canal, with ventricle below and atrium above showing labeling of the endocardial cells as a single green clone (G, green arrowhead), which gives rise to the forming vessel on the ventricle surface (G, green arrows). Part of this green clone is contiguous with the surface endothelial cells, extending through the myocardium to the AV canal and atrium (H, yellow). Analysis of multiple clones, including AV endocardium specific clones (I), shows a clonal relationship between AV endocardial cells and endothelial cells on the surface (G–J). Forming vessels can be derived from more than one clone, but always share the same labeling with the AV canal (J). Non-AV clones are found not to give rise to surface endothelium (K). Quantification of the endocardial spectral combinations found in different heart regions that match those of the vessel endothelium (L, $^*p < 0.05$ ANOVA/Tukey, ± SEM). Zebrafish age listed as dpf or italicized total body lengths with equivalent age in brackets when zebrafish were raised under non-standard conditions. AVC, atrioventricular canal; A, atrium; V, ventricle. Scale bars represent 50 μm.

Furthermore, vessel endothelial cells on the ventricle surface were often found to be contiguous with endocardial clones at the AVC (Figures 3G–3J; Figure S3M). Multiple angiogenic sprout clones were observed (an average 1.2 vessel forming clones in hearts with labeled sprouts), suggesting that multiple AV endocardial cells migrate onto the surface of the heart to form the coronary vasculature (Figure 3J; arrowhead, endocardium; arrow, angiogenic sprout). Together, these data strongly suggest that the AV canal endocardium is the major source of the angiogenic sprouts when coronary endothelial cells first emerge on the surface of the ventricle.

Coronary Vessel Formation Is Regulated by CXC Chemokines

Homeostatic chemokines have a well-established role in directing the migration and positioning of receptor expressing cells (Cha et al., 2012; David et al., 2002; Doitsidou et al., 2002; Nair and Schilling, 2008; Raz and Mahabaleshwar, 2009). CXC motif-chemokines are one such family of molecules that act on endothelial cells during angiogenesis (Ara et al., 2005; Bussmann et al., 2011; Kiefer and Siekmann, 2011; Siekmann et al., 2009; Tachibana et al., 1998; Takabatake et al., 2009). During formation of both the lateral aortae and brain capillaries, endothelial

cells expressing chemokine (CXC-motif) receptor 4a (Cxcr4a) migrate toward or over chemokine (CXC-motif) ligand 12b (Cxcl12b) expressed in the underlying endoderm and neural keel midline, respectively (Bussmann et al., 2011; Siekmann et al., 2009). During juvenile heart development in zebrafish, we found that a *cxcl12b:YFP* reporter was expressed in ventricular, but not atrial, cardiomyocytes prior to the emergence of endothelial cells onto the ventricular surface (Figure 4A; Figures S4A–S4C). This expression was maintained as the endothelial cells migrated over the ventricle (Figures 4B and 4C; Figure S4D). *cxcl12b:YFP* expression was generally observed to be strongest in the base of the ventricle in the region that tends to be vascularized first (Figures 1C, 1D, and 4D; Figures S4E and S4F). However, levels and regions of myocardial expression varied markedly between hearts (Figures 4A–4C; Figures S4A–S4D). Strong expression of *cxcl12b:YFP* was also observed in an unknown cell population on the surface of the BA prior to and after the formation of the major arterial vessel over the BA (Figures 4A–4C and 4F; Figures S4A–S4C). Following the formation of the coronary vasculature, *cxcl12b:YFP* was downregulated in cardiomyocytes, but was upregulated in epicardial derived perivascular cells which line the *kdrl:mCherry* expressing arteries (Figures 4E–4H, inset arrowheads; Figures S4G and S4H). *Tcf21:CreERt2* was used to label the epicardium in embryos (Kikuchi et al., 2011) and the descendants of these cells were found to give rise to the *cxcl12b:YFP* expressing mural cell population of arterial vessels (Figure S4I).

cxcr4a:YFP was only expressed after the emergence of endothelial cells and was more strongly expressed by migratory endothelial cells at the angiogenic sprouts (Figures 4I–4L; Figures S4J–S4L). Expression was maintained in migrating cells, but became largely downregulated as vessels formed (Figures 4K and 4L, brackets demarcate migrating cells; arrowhead, formed vessel and Figure S4M) suggesting a role for CXC signaling during the migration of these cells. Consistent with *cxcl12b:YFP* expression after vessel formation, *cxcr4a:YFP* receptor expression was maintained in endothelial cells of the mural cell-lined arteries, which express high levels of *kdrl:mCherry* and *dll4:EGFP* (Figures 4M–4P and Figures S4N–S4Q). These data suggest that Cxcr4a-Cxcl12b signaling not only guides the newly emerged endocardial derived endothelial cells, but also may later stabilize and maintain the arterial vascular network over the ventricle.

Mutant zebrafish lacking a functional Cxcr4a receptor failed to develop coronary vasculature (5 mpf, Figures 5A and 5B; Figures S5A–S5H). *cxcr4a* mutant endothelial cells migrated onto the surface of the ventricle suggesting that initiation of the sprout occurs normally, but the mutant sprouts appeared abnormal (Figures 5C–5F) and failed to migrate in a coordinated way, resulting in isolated cells or groups of cells that did not resolve to form a functional vasculature network (Figures 5A and 5B; Figures S5A–S5H). Angiographs suggested that there was no major supply of blood to the surface of the heart in the mutant other than systemic flow (Figures S5I–S5K). Mutant endothelial cells appeared to be less consolidated in the direction of their migration, simultaneously extending multiple processes in different directions (Movie S3; Figure 5G), suggesting that these endothelial cells were unable to correctly interpret the cues of their external environment. In contrast, the ligand Cxcl12b was not required for vessel formation, indicating that there may be a compensatory mechanism for coronary vessel development in *cxcl12b* mutants (Figures S6A and S6B). Evidence from other development paradigms suggests that the Cxcr4a and Cxcr4b receptors show strong affinity for Cxcl12b and Cxcl12a respectively, but in the absence of one they can respond to the other (Boldajipour et al., 2011). In addition, during fin regeneration in adult zebrafish, Cxcl12a rather than Cxcl12b appears to be the relevant ligand for Cxcr4a-dependent vascular plexus remodeling (Xu et al., 2014). Consistent with this *cxcl12a:DsRed* was indeed expressed by ventricular cardiomyocytes in *cxcl12b* mutant zebrafish (Figures S6E–S6H). To test for such compensation, *cxcl12a/b* double mutants were raised. Unlike their siblings that had at least one functional copy of *cxcl12a* or *cxcl12b*, double mutant zebrafish were found to not survive past larval stages using standard husbandry approaches (Figure S6I), suggesting broad functional redundancy between *cxcl12a* and *cxcl12b*. Using optimized husbandry (see the Experimental Procedures), a small number of *cxcl12a/b* double mutants were able to survive into juvenile stages (seven of 136 selected double mutants survived beyond 1 month, the oldest of which died at 93 dpf). Surviving double mutant zebrafish were much smaller than siblings raised under the same conditions (Figure 5H), but reached a size (>13 mm) and age at which coronary vessel development should have started. No endothelial cells, angiogenic sprouts, or formed vessels were observed on the surface of the *cxcl12a/b* double-mutant heart (Figures 5I, 5J, S5J, and S5K). Although their stunted growth could be a contributing factor to the lack vessel development in *cxcl12a/b* double mutants, some *cxcl12b−/−; cxcl12a+/−* adult fish show abnormal patterning and positioning of coronary vessels, suggesting that Cxcl12 signaling is required for proper development of the coronary vasculature (Figures S6L–S6O). Independently, Cxcl12a and Cxcr4b appeared not to be required for coronary vessel development because these mutant zebrafish developed vasculature normally (Figures S6A, S6C, and S6D).

To investigate if the underlying myocardium was using Cxcl12b-Cxcr4a signaling to provide positional information to the endothelial cells, *cxcl12b* was overexpressed in all cardiomyocytes to dilute or disrupt the local gradients and positional cues given by the endogenous ligand (Figures 6A–6F). Uniform overexpression of *cxcl12b* in the myocardium resulted in a disruption of vessel formation in juvenile zebrafish, suggesting that the location and/or dynamics of *cxcl12b* expression may be important for correct vessel formation (Figures 6A–6F). Zebrafish overexpressing *cxcl12b* had less vessel coverage between 88 and 96 dpf with endothelial cells appearing elongated or spiculated and irregularly positioned over the heart (Figures 6C–6F). Later at around 127 dpf, these overexpressing zebrafish did appear to successfully construct a vasculature network and vessel coverage becomes comparable with non-overexpressing siblings (Figure 6B; Figures S7A–S7D). It is possible that the endogenous signal reaches a level at which it can be interpreted over the exogenous signal. Ectopic expression in the atrium was insufficient to attract endothelial cells and induce vessel formation. This ectopic expression may not be strong enough to compete with the ventricular expression or additional factors may be required to vascularize the atrium (Figures S7C′–S7D′). When the developing myocardium is partially exposed to

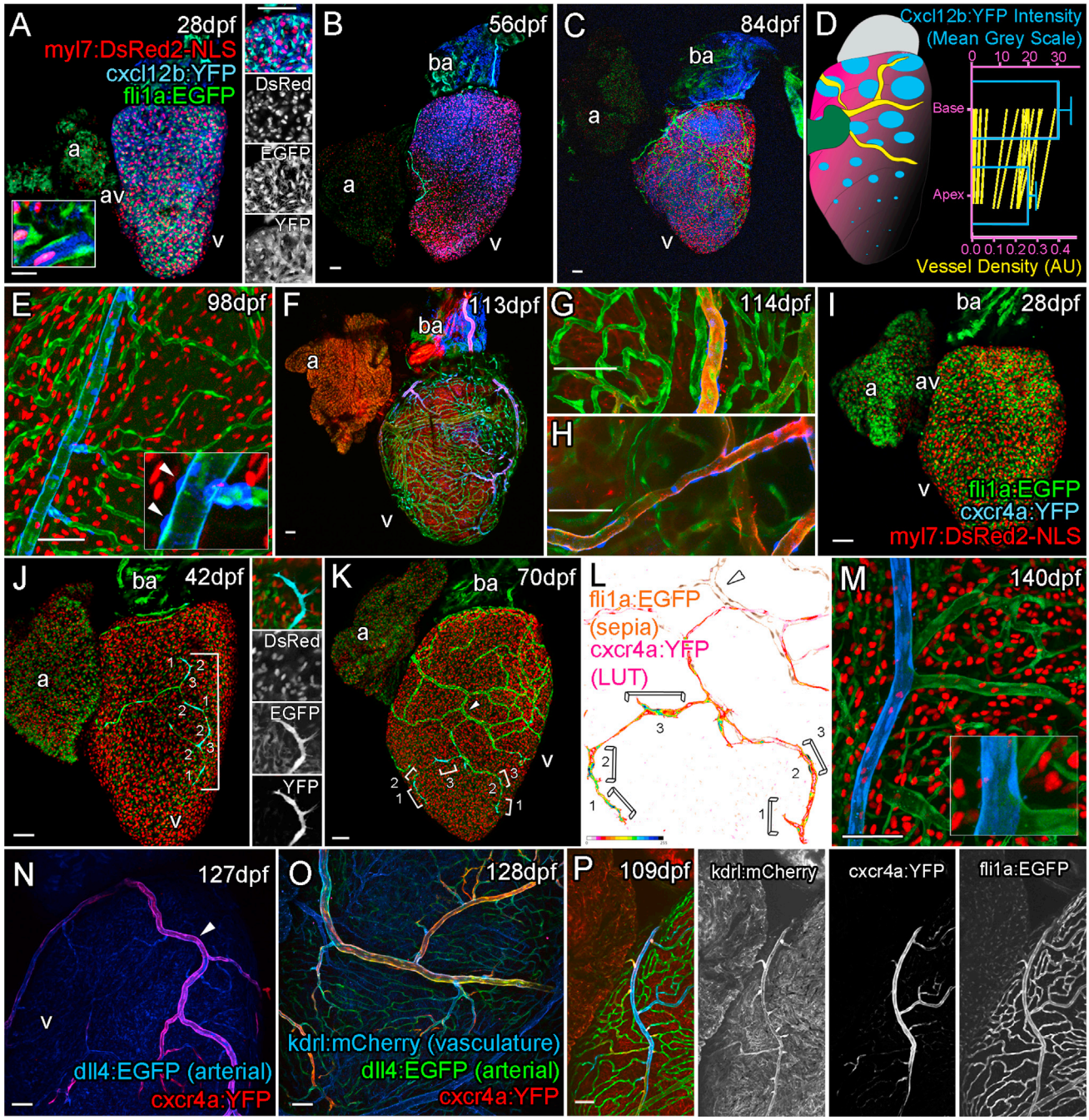

Figure 4. Cxcr4a-Cxcl12b Expression during Coronary Vessel Formation

Confocal images of transgenic fish Tg(myl7:DsRed2-NLS; fli1:EGFP; cxcl12b:YFP) (A–C, E), Tg(kdrl:mCherry; fli1:EGFP; cxcl12b:YFP) (F–H), Tg(myl7:DsRed2-NLS; fli1:EGFP; cxcr4a:YFP) (I–M), Tg(dll4:EGFP; cxcr4a:YFP) (N), Tg(dll4:EGFP; cxcr4a:YFP; kdrl:mCherry) (O), and Tg(kdrl:mCherry; fli1:EGFP; cxcr4a:YFP) (P). Ventricular, but not atrial cardiomyocytes express cxcl12b:YFP prior to and during vessel formation (A, 28 dpf; B, 56 dpf; C, 77 dpf). cxcl12b:YFP labeling is found within the cytoplasm of cells containing a myl7-positive nuclei (red) and flanked by fli1a-positive (green) endothelial cells (A, inset, from confocal section, side image). Levels of cxcl12b:YFP expression vary over the ventricle and between hearts, but are generally higher toward the base (A–D). Quantification of cxcl12b:YFP intensity (blue, normalized mean 14 dpf to 84 dpf, ± SEM) and vessel density (green, mean values of individual time points 7 dpf to 420 dpf) between base and apex of ventricular myocardium (D), both $p < 0.0001$ (Wilcoxon signed rank test on paired values). In addition, expression is observed on the surface of the bulbous arteriosus (ba, A–C, F). cxcl12b:YFP expression is downregulated following establishment of the vessel network in cardiomyocytes, but is later expressed in the mural cells of mature vessels (E, inset arrowhead). cxcl12b:YFP-expressing mural cells surround large arterial vessels (F–H, blue), which express high levels of kdrl:mCherry (F–H, red). *cxcr4a:YFP* is not expressed prior to the emergence of endothelial cells on the surface of the ventricle (I, 28 dpf). Endothelial cells of angiogenic sprouts express *cxcr4a:YFP* as they migrate over the surface of the myocardium (J, 42 dpf, image showing tip cell; K, 70 dpf; brackets label cells displaying active migration morphology; L, intensity of *cxcr4a:YFP* in endothelial cells). The majority of vessels do not express *cxcr4a:YFP* after their formation (K–P). Larger arterial vessels do maintain *cxcr4a:YFP* expression, which overlaps with high levels of *kdrl:mCherry* and *dll4:EGFP* expression in these vessels (N–P). Scale bars represent 50 μm.

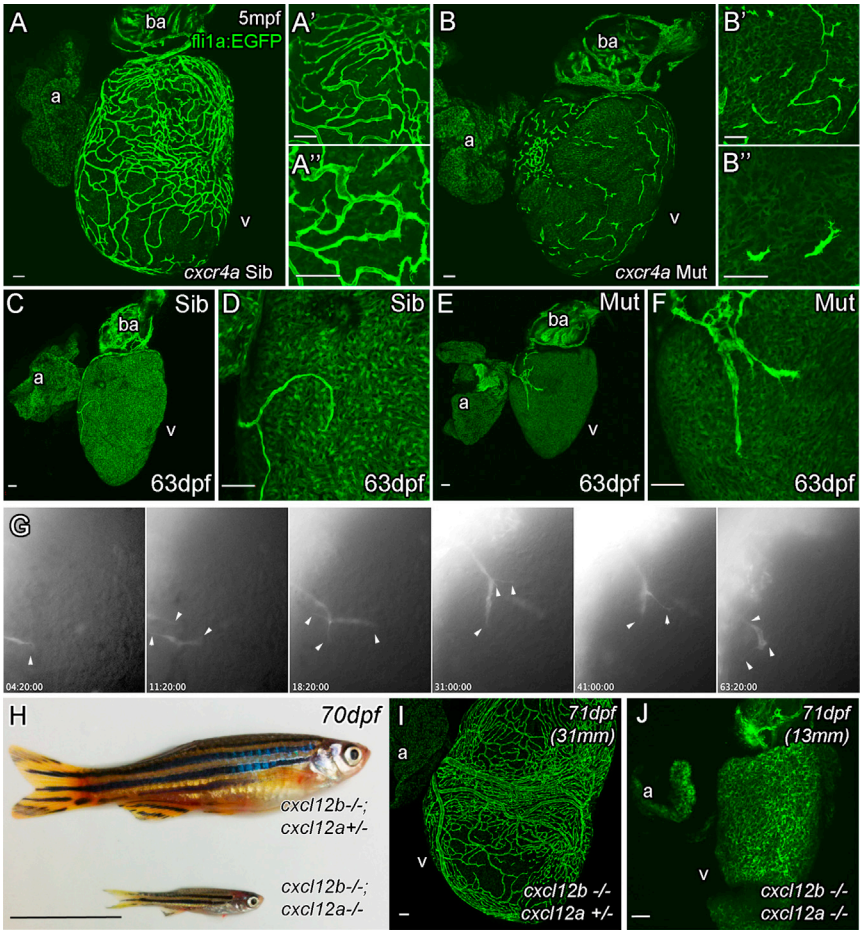

Figure 5. Cxcr4a and Cxcl12 Are Required for Coronary Vessel Formation

Endothelial cells migrate over the surface of the heart, but fail to form a vascular network in adult *cxcr4a* mutant fish (A, Sibling control and B, Mutant). As *cxcr4a* mutant endothelial cells emerge onto the surface of the heart in juvenile zebrafish, they have an uncharacteristic shape appearing truncated and highly spiculated (C–F). Movie stills show abnormal migration of endothelial cells in the mutant (G). Unlike wild-type cells, *cxcr4a* mutant cells project and retract multiple processes in different directions while migrating over the ventricle (arrowheads). They often migrate individually in different changing vectors and fail to make and maintain connections between each other. *cxcl12a; cxcl12b* double mutants show general retardation in growth in comparison with siblings raised under the same (optimized) husbandry conditions (H). Unlike their siblings, no endothelial cells are observed on the surface of heart of *cxcl12a/b* double mutant fish (I, sibling; J, *cxcl12a; cxcl12b* double mutant). Italicized age indicates zebrafish are raised using optimized husbandry, body length in brackets. Scale bars represent 50 μm, except in (H), which is 10 mm.

4OHT, it resulted in localized and stable *cxcl12b* overexpression in clonal patches of the juvenile myocardium (Figure 6G). In contrast to the disruption caused by uniform overexpression, the expression of *cxcl12b* from patches of ventricular myocardial cells resulted in greater vessel density within and around the *cxcl12b* expressing region (Figures 6G–6I; Figures S7E–S7I). Taken together, these results indicate that Cxcl12b directs the localized formation of blood vessels on the surface of the heart by directing the migration and/or retention of *cxcr4a*-positive endothelial cells.

cxcr4a Mutant Hearts Fail to Regenerate following Resection

Surprisingly, some *cxcr4a* mutant zebrafish lacking coronary vasculature are viable as adults. Although grossly normal, mutants appear to be at a competitive disadvantage, having a range of ability to adapt to the loss of coronary vessels. Following in-cross of *cxcr4a* heterozygous parents, only 16 of 294 adult offspring were found to be homozygous *cxcr4a* mutants, or 58 fewer than expected with Mendelian inheritance. Although some mutants survive, the majority of these *cxcr4a* mutant zebrafish failed to undergo heart regeneration as adults perhaps because of compromised development during juvenile stages. *cxcr4a* mutant zebrafish failed to regenerate following amputation of the apex, forming a scar instead at 30 days post-amputation (dpa; Figures 7D and 7E), 60 dpa (Figures 7A and 7B), and 120 dpa (Figures 7F and 7G). Previous studies into Cxcr4-Cxcl12 signaling during heart regeneration suggest that Cxcr4b, but not Cxcr4a, is required for correct migration and contribution of regenerating cardiomyocytes to the regenerating region (Itou et al., 2012). We demonstrated that *cxcr4a* is required during development; our results suggest that developmental disruption of coronary vascularization results in a zebrafish heart that is unable to respond to injury in the typical manner.

DISCUSSION

Here we have described for the first time the vascularization of the ventricle in zebrafish by angiogenesis of endothelial cells from the endocardium. The presence of coronary vessels in all studied elasmobranchs suggests they have origins early in vertebrate heart evolution coinciding with the emergence of compact myocardium (Sedmera and Wang, 2012). Consistent with this, in zebrafish only the ventricle becomes vascularized after the compact myocardium has formed post-embryonically and the more spongy and thinner-walled atrium never develops coronary vessels (Figure 1; Figures S3P and S3Q). Unlike mammalian cardiac muscle, the fish myocardium is not strictly dependent on coronary circulation for survival (Figure 5). During evolution, coronary vessels have been lost in many teleost species, and some amphibians have only a vestigial non-penetrating coronary vessel on the surface of the outflow tract (Sedmera and Wang, 2012). It appears that coronary vessels evolved initially as a supplement of cardiac oxygen supply, but later became strictly required for the function and survival of the myocardium. Our

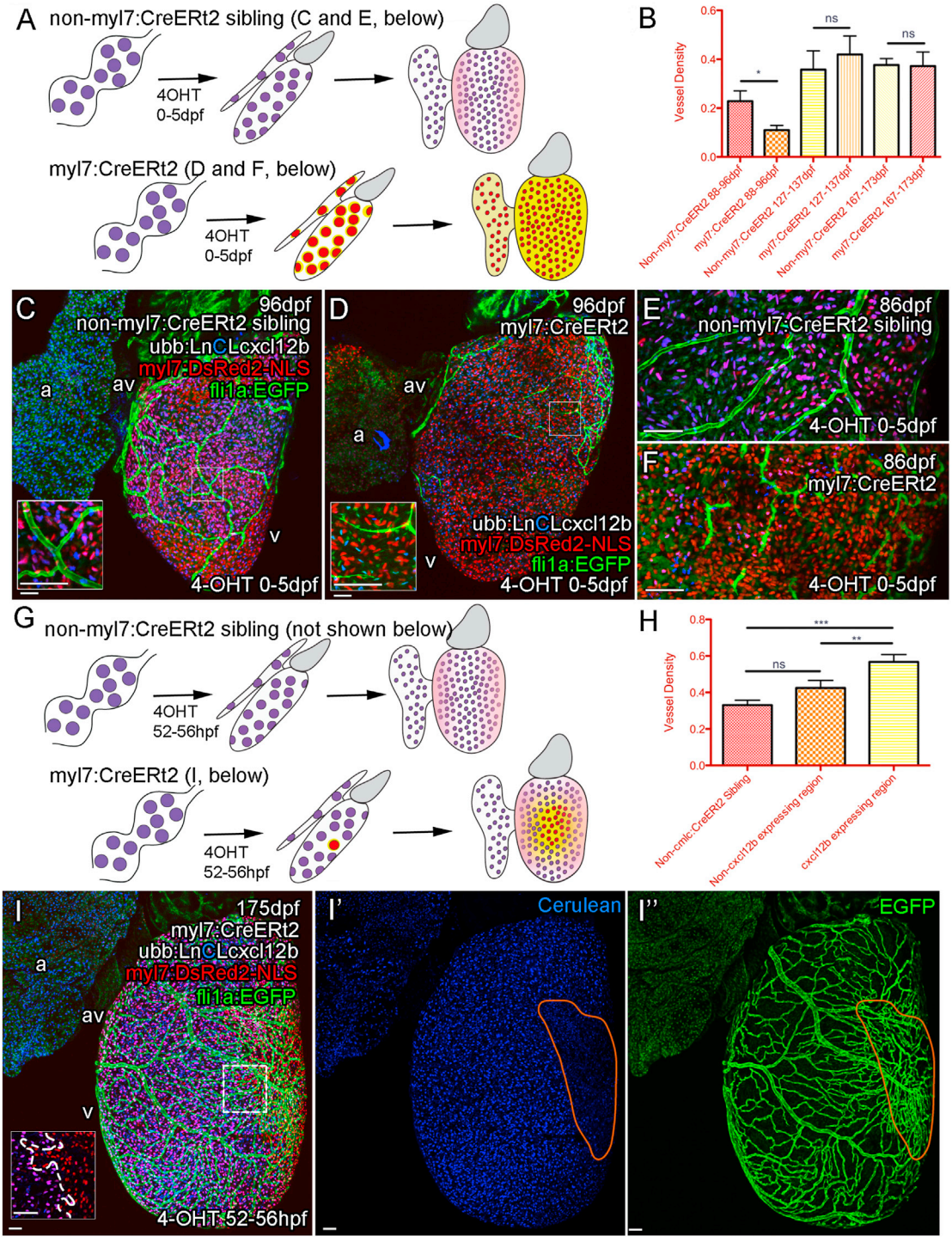

Figure 6. Cxcl12b Likely Provides Positional Information for Coronary Endothelial Cells

(A–E) Uniform overexpression of Cxcl12b in quadruple transgenic fish *Tg(Ubb:LoxPH2B-Cerulean-LoxP-cxcl12b(ubb:LnCLcxcl12b); myl7:DsRed2-NLS; fli1a:EGFP; myl7:CreERt2)*. Schematic illustration of uniform overexpression of Cxcl12b. Prolonged treatment with 4OHT between 0 and 5 dpf resulted in efficient removal of the floxed H2B-Cerulean (blue) and expression of *cxcl12b* (yellow) in *myl7*-positive cardiomyocytes (red) such that any endogenous Cxcl12b signaling pattern may be obscured (pink) (A). Such overexpression of *cxcl12b* in cardiomyocytes also resulted in an initial disruption of vessel formation (B, quantification of vessel density, *p < 0.05 t test, ± SEM; C, non-*myl7:CreERt2* sibling control; D, *cxcl12b* overexpression) with endothelial cells becoming elongated and appearing spiculated (C and D, inset, E [control], and F [overexpression]). (G–I) Clonal overexpression of Cxcl12b in quadruple transgenic fish *Tg(Ubb:LoxPH3B-Cerulean-LoxP-cxcl12b (ubb:LnCLcxcl12b); myl7:DsRed2-NLS; fli1a:EGFP; myl7:CreERt2)*. A 4-hr pulse of 4OHT results in partial activation of the *myl7:CreERt2* transgene prior to cardiomyocyte clonal expansion. *cxcl12b* is then expressed from a localized source in adult zebrafish (G, I; inset from box; boundary between

(legend continued on next page)

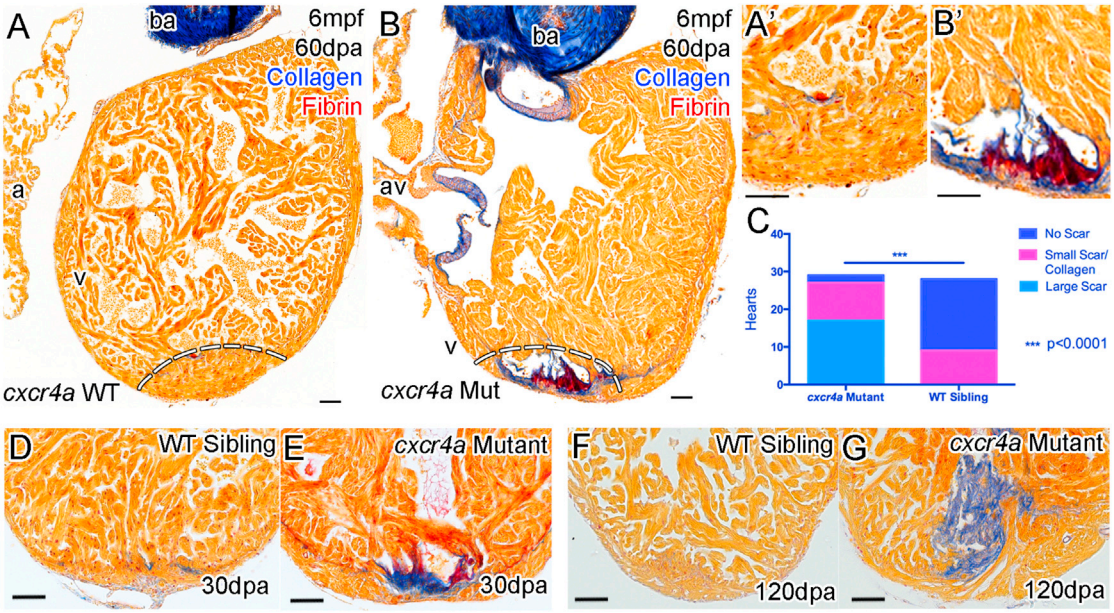

Figure 7. *cxcr4a* Mutant Hearts Fail to Regenerate following Resection of the Ventricle Apex

Zebrafish with functional Cxcr4a-Cxcl12b signaling and normal coronary vessel development completely regenerate resected ventricle tissue after 60 days (A, dashed line represents amputation plane, and A′). Adult *cxcr4a* mutants with disrupted coronary vessel development fail to regenerate the resected tissue resulting in the deposition and persistence of collagen (blue) and fibrin (red) (B, B′, E, and G). Quantification of scar formation at 60 dpa (C). *cxcr4* mutant zebrafish have significantly more collagen deposition and scar formation compared to the siblings (***$p < 0.0001$, chi-square). Scar formation is observed by 30 dpa (D and E) and persists beyond 120 dpa (F and G) with no apparent effect on survival. Scale bars represent 50 μm.

study suggests that angiogenesis could be the ancestral method of coronary vessel development and this mechanism is at least partially conserved in mammals (Chen et al., 2014; Red-Horse et al., 2010; Tian et al., 2013).

Divergent cell populations have been identified as the source of coronary vascular endothelial cells (Chen et al., 2014; Pérez-Pomares et al., 2002; Red-Horse et al., 2010; Tian et al., 2013, 2014; Wu et al., 2012). Our results suggest that zebrafish coronary endothelial cells derive from the endocardium at the atrioventricular canal. Although the source of coronary vascular endothelial cells remains unclear in elasmobranchs such as the dogfish (Muñoz-Chápuli et al., 1996), the process of coronary vascular development is thought to be initialed by the formation of a diverticulum from the SV (De Andrés et al., 1993). This is consistent with observations in mice demonstrating the SV endocardium as a major source of vascular endothelial cells (Chen et al., 2014; Red-Horse et al., 2010; Tian et al., 2013). Interestingly, the site of the future coronary sinus in both dogfish and mice may match this point of origin (the SV becoming atrialized in mammals). In contrast, zebrafish appear to lack such a coronary sinus on the SV and this chamber also seems to largely lack cardiomyocytes (Figures S3P and S3Q). Furthermore, both the SV and the atrium remain unvascularized in adult zebrafish, and at no point did we observe endothelial cells on their surface during development (Figure 1; Figures S3P and S3Q). In addition, observed early angiogenic sprouts on the surface of the heart appear to be arterial (as are the endocardial endothelial cells) rather than venous (Figure 1). Taken together, we think it unlikely that the SV has a major direct contribution to the developing vasculature. However, earlier developmental contributions to the endocardium of the AV canal from the SV, proepicardium, or liver primordium in zebrafish is still possible (Katz et al., 2012; Poelmann et al., 1993; Tian et al., 2013).

Given the evolutionary distance between dogfish and zebrafish it is conceivable that such anatomical changes could occur and this reinforces the notion that there is a degree of developmental plasticity for the coronary vasculature. Nonetheless, in these species, coronary vessel formation remains fundamentally consistent with what is known to occur in mammals, in which it develops from existing endothelial cell populations residing within the heart (Chen et al., 2014; Red-Horse et al., 2010; Tian et al., 2013, 2014; Wu et al., 2012). The ventricular endocardium has also been shown to give rise to coronary endothelial cells during postnatal trabecular compaction in the mouse inner myocardial wall (Tian et al., 2014). The zebrafish myocardium remains trabecular and the coronary vasculature remains superficial in subepicardial space, further suggesting that coronary vasculature formation is a versatile process that evolves responsively to fulfill the need of myocardium.

exogenous-*cxcl12b* expressing [DsRed-NLS positive] and non-expressing [H2B-Cerulean and DsRed-NLS double positive] cardiomyocytes is marked with a dashed line). There is an increase in density of vessels over and proximal to the region of *cxcl12b* expressing cardiomyocytes (H, I, I′, and I″, region of *cxcl12b*-expressing (Cerulean-negative) cardiomyocytes demarcated by orange). (H) Quantification of vessel density. Representative image of clonal overexpression (I, I′, and I″) with region of cxcl12b-expressing (Cerulean-negative) cardiomyocytes demarcated by orange. **$p < 0.01$; ***$p < 0.0001$ t test. Scale bars represent 50 μm.

Observations in birds led to the initial hypothesis that the major mode of coronary vessel formation was by vasculogenesis of epicardial-derived cells (Pérez-Pomares et al., 2002). This is inconsistent with a number of our observations in zebrafish including the nature of endothelial cell migration (Movie S1); the progressive formation of vessels over the heart (Figure 1); the continuous lineage-based labeling along vessels derived from *fli1a*-expressing endothelial cells (Figure 3); and the clonal nature of the early angiogenic sprouts on the ventricle surface (Figure 3). The epicardium does, however, provide a source of vascular support cells to the coronary arteries that express *cxcl12b* and may have an important role in maintaining the integrity of the coronary vasculature (Figures S1 and S4) (Kikuchi et al., 2011).

Although the coronary vasculature is a supplemental cardiac oxygen supply, it has an important function in zebrafish. Most *cxcr4a* mutants lacking coronary vessels do not survive to adulthood, but those that do survive appear to lack the normal regenerative response to injury (Figure 7). What physiological and developmental adaptations take place in the zebrafish myocardium in response to the loss of this oxygen supply remains to be elucidated. How these factors impinge on the regenerative process are fascinating areas of future study and is of relevance in patients whose coronary vessel function in is compromised by occlusion or abnormal development. Understanding the underlying molecular and cellular basis of these adaptations will prove beneficial to patients whose endogenous regenerative responses to myocardial damage could be harder to stimulate by clinical intervention. Furthermore, enhancing collateral blood vessel formation by Cxcl12/Cxcr4 guided angiogenesis might be beneficial after ischemic heart injuries.

Given the medical burden associated with compromised coronary vasculature function, finding new avenues of therapy for coronary heart disease is imperative (Alwan, 2011; Taylor et al., 1992). Stimulating endogenous endothelial cells with exogenous factors or introducing analogous cells is a promising strategy for alleviating pathologies associated with coronary artery disease and malformation. Here we have identified CXC chemokines as one such potential exogenous factor. As discussed, *cxcr4a* is required for vessel formation in juvenile zebrafish (Figure 5) and modulation of *cxcl12b*-expression can influence the formation and density of coronary vessels (Figure 6). Interestingly an SNP in the region of human CXCL12 has consistently been associated with early onset myocardial infarction and coronary artery disease in GWA studies of patients, suggesting that the mechanisms uncovered here in zebrafish may be conserved in humans and gives promise to their effective modulation in a clinical setting (Kathiresan et al., 2009; Samani et al., 2007; Schunkert et al., 2011).

EXPERIMENTAL PROCEDURES

Zebrafish Strains

Tg(fli1a:EGFP)^{y1}; *Tg(fli1a.ep:DsRedEx)*^{um13} (referred to as *fli1a:DsRed*); *Tg(−5.1myl7:DsRed2-NLS)*^{f2}; *Tg(kdrl:GRCFP)*^{zn1}; *Tg(−6.5kdrl:mCherry)*^{ci5}; *Tg(−3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701}; *Tg(cryaa:DsRed,-5.1myl7:Cre-ERT2)*^{pd10} (referred to as *myl7:CreERt2*); *TgBAC(tcf21:DsRed2)*^{pd37}; *TgBAC (cryaa:EGFP,tcf21:Cre-ERT2)*^{pd42} (referred to as *tcf21:CreERt2*); *Tg(cxcl12a:DsRed2)*^{umn1}; *Tg(wt1b:EGFP)*^{li1}; *Tg(Mmu.Dll4F2-ADV.E1B:EGFP)*^{sgf1} (referred to as *dll4:EGFP*); *Tg(−0.8flt1:RFP)*^{hu5333} (referred to as *flt1:tdtomato*); *TgBAC(flt4:Citrine)*^{hu7135} (referred to as *flt4:YFP*); *TgBAC(prox1aBAC:KalTA4-4xUAS-ADV.E1b:TagRFP)*^{nim5} (referred to as *prox1a:RFP*); *Tg(ubb:LOX2272-LOXP-RFP-LOX2272-CFP-LOXP-YFP)*^{a132} (referred to as *ubb:zebrabow*); and *Tg(kdrl:Cre-ERT2)*^{fb13} transgenic lines have been described previously (Bussmann et al., 2010; Cross et al., 2003; Glass et al., 2011; Kikuchi et al., 2010, 2011; Lawson and Weinstein, 2002; Mably et al., 2003; Mosimann et al., 2011; Pan et al., 2013; Perner et al., 2007; Proulx et al., 2010; van Impel et al., 2014; Wythe et al., 2013; Zhao et al., 2014), as have mutant lines *cxcr4a*^{um20}, *cxcl12b*^{mu100}, *cxcr4b*^{t26035}, *cxcl12a*^{t30516} (Bussmann et al., 2011; Knaut et al., 2003; Siekmann et al., 2009; Valentin et al., 2007). Details of zebrafish lines not previously described, along with husbandry and Cre induction protocols, are described in the Supplemental Experimental Procedures.

Confocal Imaging

Hearts were isolated from terminally anesthetized zebrafish, rinsed in PBS, and fixed briefly (30 s) in 4%PFA/PBS. Whole isolated hearts were then mounted in 1% low-melting point agarose/PBS in a glass-bottom dish for confocal microscopy. A Z-stack of both restricted band-pass channel and lambda images was then acquired using LSM700 and LSM710 microscopes (Ziess). Coronary vessel, endocardial patch, and ventricle area was calculated from max projected confocal images of the hearts using ImageJ/Fiji; surface volume and contiguous pixel analysis was carried out using IMARIS software. The ventricle or individual patches were traced and pixels counted using differential threshold limits to measure surface vessel endothelial cell, endocardial patch, and total area. Quantification of *cxcl12b:YFP* intensity was carried out using the mean gray value measurement function in ImageJ/Fiji outlining the base for the ventricle (defined as the area above the AVC connection) and apex (below the AVC connection) and normalizing both to the (*cxcl12b*-negative) atrium. Quantification of *cxcr4a:YFP* intensity was carried using a 16-color look up table calibrated across the intensity spectrum of *cxcr4a:YFP* in ImageJ/Fiji.

Heart Explant Culture and Live Imaging

Hearts were isolated from terminally anesthetized *fli1a:EGFP* transgenic zebrafish and placed in Ringer's solution containing 100 μg/ml Primocin and 150 U/ml heparin and transferred to glass-bottom dishes containing L-15 media supplemented with 10% FCS, 100 μg/ml Primocin, 1.25 mM $CaCl_2$, and 800 mg/l glucose. Hearts were then imaged using a Leica DM IRE2 equipped with a 20×/0.40 objective, Leica GFP filter cube (ex470/40, em525/50) and a Hamamatsu ORCA-ER camera. μManager acquisition software (Edelstein et al., 2010) is used to capture a predefined z stack of images every 20 min over 4 days. As the heart was still beating, images were selected that contained the area of interest within the focal plane for each given time point and arranged to form a time-lapse movie.

Amputation, Angiography, and AFOG

Ventricular amputations and adult zebrafish angiography were carried out as previously described (Poss et al., 2002; Pugach et al., 2009) and both in accordance with CHLA IACUC animal care protocols. Tissue collection, preparation, and histological AFOG (Acid Fuchsin Orange-G) were carried out using standard protocols (Poss et al., 2002).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.04.001.

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